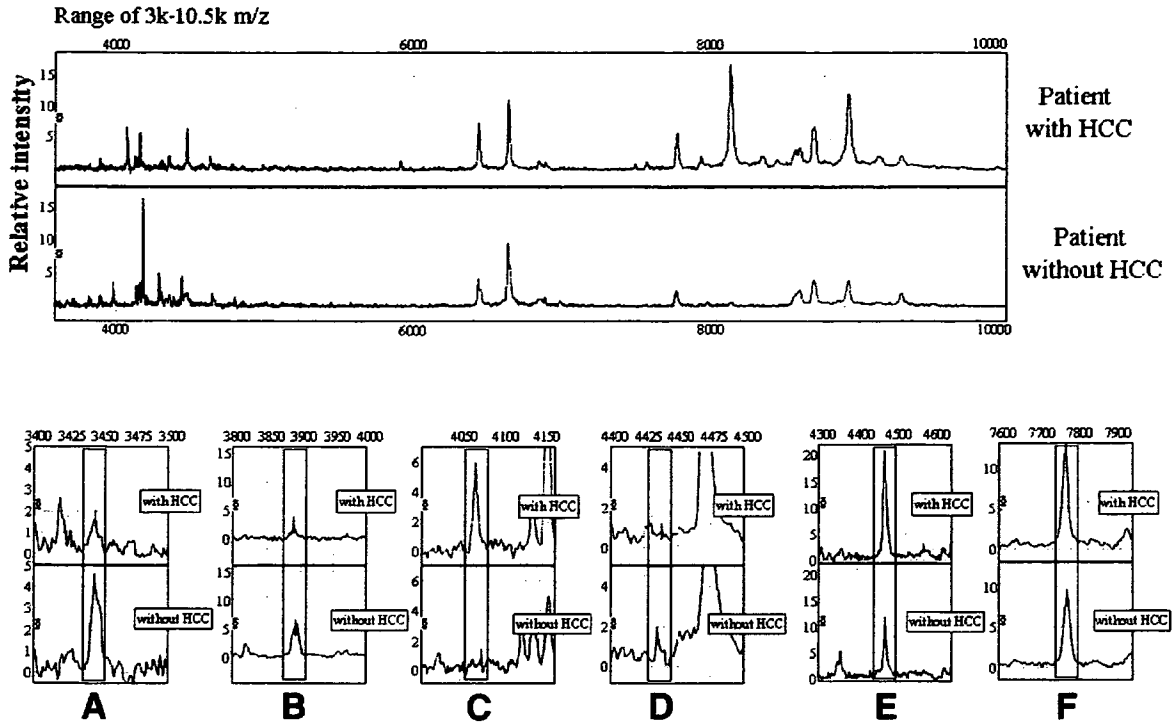

Fig. 1. Analysis of sera from patients with and without HCC (spectrum). Serum samples were applied to CM10 ProteinChip Arrays. Representative spectra from patients in each of the 2 groups (HCC and non-HCC) are presented. The horizontal axis indicates protein mass to charge (m/z), whereas the longitudinal axis designates the relative intensity. Lower highlight panels represent the peaks used in the classifier described in Fig. 2. Peaks of (A) 3444, (B) 3890, (C) 4067, (D) 4435, (E) 4470, and (E) 7770 m/z are shown.

second test set (3rd analysis group) of samples taken from 7 patients 1 year before the development of HCC and 5 patients with chronic liver disease remaining free of HCC for at least 3 years. Six of the 7 (86%) patients who later developed HCC were classified to the HCC group using the classifiers described previously (Fig. 2, Table 3), even though the HCC was undetectable by US at the time of serum testing. All 5 patients without HCC were classified to the non-HCC group. These results indicate that this decision tree analysis is useful for the early diagnosis of HCC.

Discussion

Proteomic analyses of sera and liver tissues from patients with HCC associated with HBV or HCV infection has been used to identify new biomarkers predicting HCC development, leading to improved prognosis.[21-27] Because many analyses use 2-dimensional electrophoresis, the proteins used in such investigations must typically be greater than 10,000 daltons in molecular weight.[21,25-29] Analyzing serum or another body fluid that is easy to obtain from patients to predict disease or evaluate treatment efficacy would be ideal. In this study, we used the SELDI ProteinChip system to analyze serum samples from patients with HCC. This affinity-based mass spectrometric method, which combines chromatography and MS, is suitable for the analysis of both proteins and low-molecular-weight peptides.[14] Although we did not identify a single effective biomarker, we developed a new decision tree, using a cross-validation approach, that uses a multimarker algorithm of 6 proteins capable of diagnosing and predicting HCC at least 1 year before the appearance of clinically detectable disease in patients infected with HCV.

Ninety percent of the protein content of serum is composed of 10 proteins, including albumin and IgG; an additional 12 proteins make up 90% of the remaining 10%. Thus, only 1% of the protein content of serum is of interest as potential biomarkers in proteomic studies.[30] Several proteomic methods combine high-resolution separation of complex protein mixtures with additional protein identification methods, such as MS. To identify the low abundance proteins of interest, one must remove the most abundant proteins from the serum by techniques such as immunodepletion. These methods are only reliable if the assumption that biomarkers are not bound to major circulating proteins is correct. If bound to these proteins, low-abundance biomarkers would be lost by im-

Table 3. Discriminatory Peaks and Mean Values Between Groups (HCC* and Non-HCC Group)

m/z	HCC (n = 35)	Non-HCC (n = 44)	p value
Overexpressed proteins			
4067†	3.94 ± 4.56	1.92 ± 1.79	0.03
4470†	8.36 ± 4.28	6.49 ± 3.99	0.01
6433	13.61 ± 10.10	8.94 ± 8.42	0.02
6632	26.87 ± 18.11	18.20 ± 15.09	0.02
7770†	8.40 ± 5.94	5.26 ± 4.42	0.0002
8138	12.76 ± 14.78	5.86 ± 5.37	0.006
8605	4.39 ± 3.08	3.20 ± 2.45	0.02
8934	16.10 ± 10.69	10.36 ± 7.26	0.009
Downregulated proteins			
3326	1.27 ± 0.74	2.10 ± 1.21	0.003
3398	0.90 ± 0.77	2.43 ± 2.50	0.0008
3444†	2.02 ± 1.18	2.45 ± 1.50	0.2
3816	1.98 ± 1.17	3.45 ± 2.84	0.002
3826	1.65 ± 4.95	2.51 ± 3.53	0.002
3890†	3.12 ± 1.35	3.31 ± 1.41	0.2
4135	3.45 ± 2.24	5.08 ± 3.86	0.01
4175	5.49 ± 9.46	12.32 ± 14.63	0.001
4435†	1.23 ± 1.73	2.31 ± 2.63	0.006
4658	1.14 ± 0.80	1.94 ± 1.71	0.007
4791	2.42 ± 1.33	4.04 ± 3.27	0.004
6979	0.82 ± 0.52	1.19 ± 0.67	0.01

NOTE. Data are shown as the means±SD, statistical differences were determined using the Mann-Whitney U test, †Peaks selected in final classification model by decision tree analysis.

Abbreviation: *hepatocellular carcinoma.

munodepletion techniques, leading to the loss of valuable diagnostic information.[31] Therefore, we did not remove major serum proteins (albumin and IgG) from this study; analysis using the SELDI ProteinChip system can be performed without immunodepletion.

The characteristics of patients such as sex and age, sample collection method, processing and storage of samples, and data analysis methods may induce bias into proteomics-based biomarker discovery attempts. Because HCC occurs more frequently in males than females, we developed our classification model using male patients only. As a result, our study was not designed to address the benefit of our classification model for females with HCC. Villanueva et al.,[32] however, reported that gender did not appear to affect the peptide profile. We also evaluated five female patients with HCC; the peak intensity at 8136 m/z was elevated to a similar degree as that seen in male patients with HCC. Currently, a prospective study of female patients with or without HCC is underway to validate the utility of this classification model as a marker for the detection of HCC, particularly at early stages.

We demonstrated that 18 of the selected 55 protein peaks within a m/z range of 3000 to 10,500 range differed between patients with and without HCC by univariate analysis. Based on the peak intensities of the 55 peak proteins, 6 peaks were selected to construct the decision tree for the first analysis group using Biomarker Patterns Software and a 10-fold cross-validation approach. Two (3444 and 3890 m/z peaks) of those 6 peaks, however, were not significantly different between the HCC and non-HCC groups by univariate analysis (P values of 0.2, Table 3). The selection process to construct the decision tree was not based on univariate analysis; the presented decision tree was developed using multivariate binary logistic regression to determine the peaks best able to differentiate patients with and without HCC.[19,33] In fact, the ROC AUC of each of these 6 peaks were between 0.61 and 0.71, which tended to be more discriminatory than other serum markers. The decision tree proved to be best able to predict the presence of HCC in comparison with other serum markers. For these reasons, analysis of all 6 peaks, including the 2 peaks that were not significantly different between patients with and without HCC (peaks at m/z = 3444 and 3890), had the highest discriminatory power.

The algorithm used in this study is well established as a diagnostic tool for malignant neoplasms.[13,16,34,35] In comparison with the use of a single biomarker for the diagnosis of disease, multiple-biomarker analysis has both higher sensitivity and specificity. Indeed, our multimarker analysis was more accurate than existing tumor marker analysis methods (Table 4). Multimarker analysis is useful to predict HCC in patients with liver cirrhosis, which has high malignant potential and heterogeneous characteristics. Complex serum proteomic patterns may reflect the underlying pathological state of an organ, including

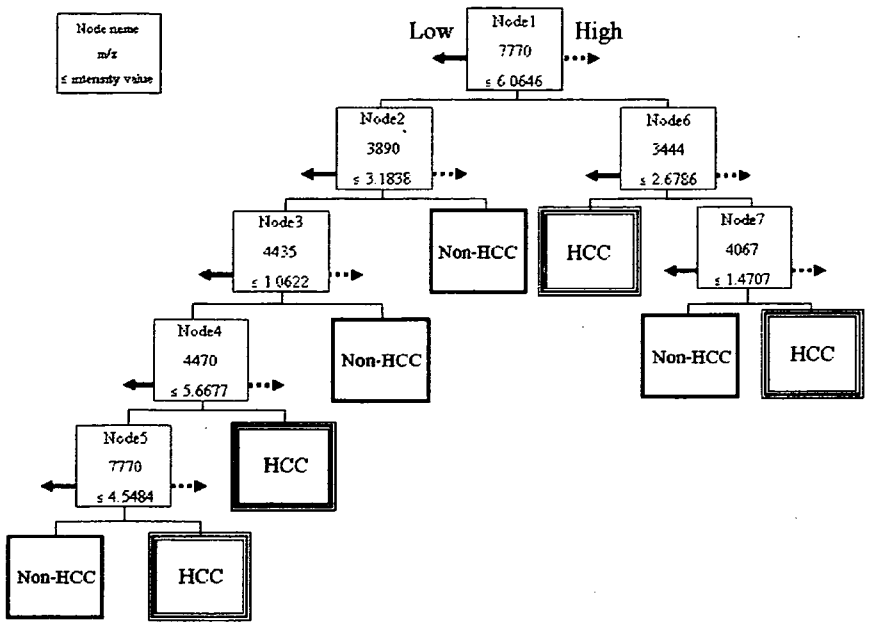

Fig. 2. Classification of HCC and non-HCC samples in the first analysis group. The decision tree was constructed using serum samples from 79 patients. The classification of a particular pattern began at the roof node, following the appropriate links based on the answer to the question at each node. If the peak 1 intensity was higher, the right node was selected. If the peak 1 intensity was lower or equal, the left node was selected. This process was repeated until a terminal node was reached. The decision tree was constructed to correctly classify 97% of the HCC samples in the first analysis group. The upper, middle, or lower lines in the box indicate the node name, molecular weight, and intensity value, respectively.

HCC. Recently, Schwegler et al.[16] reported an algorithm using the seven peaks that scored highest by SELDI TOF/MS. The determined classification tree, however, could not distinguish HCC from chronic liver disease; using 38 SELDI peaks, the sensitivity and specificity (61% and 76%) for distinguishing chronic HCV from HCV-HCC were lower than those determined for the decision tree constructed in this study. Schwegler et al. demonstrated that their sensitivity and specificity values increased to 75% and 92%, respectively, when AFP/DCP/GP73 was added to their classification model. In our model, although the sensitivity increased to 92%, specificity did not increase (52%) after the addition of AFP/AFP-L3/DCP to our classification. Serum GP73 levels, which were not available for examination in our study, or other as-yet-unknown characterizations of these patients may affect the predictive capability of this method. Although the sensitivity and specificity (92% and 90%) of another proteomics study using SELDI to distinguish chronic liver disease from HCC were higher than those determined in our study, greater than 63% of the study population examined exhibited advanced HCC (stage III and IV).[16,36] Only 14% of the HCC patients included in our study population had stage III or IV disease (Table 1), which likely accounts for the differences in the peaks used in the 2 studies. The characteristics of the patients with HCC will likely affect both the sensitivity and specificity significantly. Thus, our decision tree is more suitable for the diagnosis of early HCC than any previously reported methods.[16,36]

Although serum AFP level greater than 400 ng/ml serves as a useful method for the diagnosis of HCC,[37] this detection method is insufficiently sensitive to detect small HCCs.[38] Although the utility of several other markers has been shown to be superior to AFP in detecting early HCC,[22,39,40] these markers were determined in patients with clinically apparent HCC. Thus, the sensitivity/specificity also may not be sufficient to detect early HCC. Our classification tree was able to predict cancer occurrence before HCC was clinically apparent by US. In the third analysis group, we correctly predicted the progression of 86% of the patients to HCC from their prediagnostic

Table 4. Comparisons of Hepatocellular Carcinoma Diagnostic Rates for the Multiple Marker and Three Additional Tumor Marker Analyses in the Second Analysis Group

Markers	Sensitivity	Specificity	ROC AUC****
Multiple-marker	83% (24/29)	76% (25/33)	0.79
AFP* (>20 ng/mL)	41% (12/29)	67% (22/33)	0.57
AFP-L3** (>15%)	17% (5/29)	88% (29/33)	0.56
DCP†,*** (>40 mAU/mL)	39% (11/28)	81% (26/32)	0.64

NOTE. †excluding subject whose data could not be obtained.

Abbreviation: *alpha fetoprotein, **Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein, *** des-γ-carboxy prothrombin, ****receiver operating characteristic area under the curve.

serum samples. To screen high-risk patients with chronic liver disease, such as that associated with HCV infection, our multi-marker analysis could help distinguish those patients for which the combined examination of US, CT, and arterial portography would be recommended.

In their investigation of differential protein expression in HBV-associated and HCV-associated HCC, Kim et al.[26] identified 60 proteins displaying significant changes in expression levels between nontumorous and tumorous tissues. Forty-six of these proteins demonstrated an association with viral infection. We analyzed the sera of patients with HBV-associated HCC; the expression of a number of protein markers differed between HCV and HBV infections (data not shown). The biological and pathogenic activities of these 2 viruses are different; the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis also may differ between HBV and HCV infections.[26,41] Our analysis of the proteome using the SELDI technique demonstrates that this methods also may be useful for investigation of the molecular mechanisms of hepatocarcinogenesis on the background of different viral infections.

A number of the peaks may represent doubly charged peaks; for example, the peak at 4067 m/z may be the doubly charged form of the 8138-m/z peak. One of the peaks in Table 3 included in the classification model also may be a doubly charged peak (3890/7770 m/z), which could affect the independent variables. To clarify this possibility, one must identify the individual proteins. The major limitation of the SELDI technique is that identification of individual proteins is often complicated. Lee et al.,[42] however, recently isolated complement C3a as a candidate biomarker in human chronic hepatitis C and HCV-related HCC using the SELDI-TOF MS system after serum fractionation, 2-dimensional gel electrophoresis, in-gel digestion, and MS. We are now identifying the single protein represented by the 8138-m/z peak; 3 candidate proteins are known. Although we have to confirm these results by western blotting, the peak at 4067 m/z does not appear to be the doubly charged peak of the 8138-m/z peak by SELDI immunoassay. Although the serum levels of no single protein are sufficient to detect early HCC from the results of ROC AUC, identification of proteins altered in the disease may help analyze the molecular mechanisms underlying HCC development and may help identify new therapeutic targets or modalities for the treatment or prevention of HCC.

In patients with HCV infection, serum profiling using the SELDI ProteinChip system is useful both for the early detection of HCC and to distinguish HCC from chronic liver disease in the absence of HCC. Our ability to identify proteomic alterations in serum samples from HCC patients suggests that the SELDI ProteinChip system may be useful to identify proteins associated with HCC in the hopes of developing new therapeutic targets.

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References

1. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745-750.
2. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med 1999;131:174-181.
3. Heathcote EJ. Prevention of hepatitis C virus-related hepatocellular carcinoma. Gastroenterology 2004;127:S294–302.
4. Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. HEPATOLOGY 1994;19:61-66.
5. Ishii M, Gama H, Chida N, Ueno Y, Shinzawa H, Takagi T, et al. Simultaneous measurements of serum alpha-fetoprotein and protein induced by vitamin K absence for detecting hepatocellular carcinoma. South Tohoku District Study Group. Am J Gastroenterol 2000;95:1036-1040.
6. Okuda H, Nakanishi T, Takatsu K, Saito A, Hayashi N, Takasaki K, et al. Serum levels of des-gamma-carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathologic features of solitary hepatocellular carcinoma. Cancer 2000;88:544-549.
7. Grazi GL, Mazziotti A, Legnani C, Jovine E, Miniero R, Gallucci A, et al. The role of tumor markers in the diagnosis of hepatocellular carcinoma, with special reference to the des-gamma-carboxy prothrombin. Liver Transpl Surg 1995;1:249-255.
8. Wang CS, Lin CL, Lee HC, Chen KY, Chiang MF, Chen HS, et al. Usefulness of serum des-gamma-carboxy prothrombin in detection of hepatocellular carcinoma. World J Gastroenterol 2005;11:6115-6119.
9. Marrero JA, Su GL, Wei W, Emick D, Conjeevaram HS, Fontana RJ, et al. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in american patients. HEPATOLOGY 2003;37:1114-1121.
10. Taketa K, Okada S, Win N, Hlaing NK, Wind KM. Evaluation of tumor markers for the detection of hepatocellular carcinoma in Yangon General Hospital, Myanmar. Acta Med Okayama 2002;56:317-320.
11. Khien VV, Mao HV, Chinh TT, Ha PT, Bang MH, Lac BV, et al. Clinical evaluation of lentil lectin-reactive alpha-fetoprotein-L3 in histology-proven hepatocellular carcinoma. Int J Biol Markers 2001;16:105-111.
12. Oka H, Saito A, Ito K, Kumada T, Satomura S, Kasugai H, et al. Multicenter prospective analysis of newly diagnosed hepatocellular carcinoma with respect to the percentage of Lens culinaris agglutinin-reactive alpha-fetoprotein. J Gastroenterol Hepatol 2001;16:1378-1383.
13. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, et al. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 2002;62:3609-3614.
14. Paradis V, Degos F, Dargere D, Pham N, Belghiti J, Degott C, et al. Identification of a new marker of hepatocellular carcinoma by serum protein profiling of patients with chronic liver diseases. HEPATOLOGY 2005; 41:40-47.
15. Uto H, Hayashi K, Kusumoto K, Hasuike S, Nagata K, Kodama M, et al. Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperedemic area of Japan. Hepatol Res 2006;34: 28-34.

16. Schwegler EE, Cazares L, Steel LF, Adam BL, Johnson DA, Semmes OJ, et al. SELDI-TOF MS profiling of serum for detection of the progression of chronic hepatitis C to hepatocellular carcinoma. HEPATOLOGY 2005;41: 634-642.
17. Duda RO, Hart PE, Stork DG. Pattern classification. 2nd ed. Hoboken, NJ: Wiley-Interscience, 2001.
18. Scarlett CJ, Saxby AJ, Nielsen A, Bell C, Samra JS, Hugh T, et al. Proteomic profiling of cholangiocarcinoma: diagnostic potential of SELDI-TOF MS in malignant bile duct stricture. HEPATOLOGY 2006;44:658-666.
19. Ambroise C, McLachlan GJ. Selection bias in gene extraction on the basis of microarray gene-expression data. Proc Natl Acad Sci U S A 2002;99: 6562-6566.
20. Breiman L, Friedman JH, Olshen RA, Stone CJ. Classification and regression trees. Belmont, CA: Wadsworth International Group, 1984.
21. Lim SO, Park SJ, Kim W, Park SG, Kim HJ, Kim YI, et al. Proteome analysis of HCC proteome analysis of hepatocellular carcinoma. Biochem Biophys Res Commun 2002;291:1031-1037.
22. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: A novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125:89-97.
23. Hippo Y, Watanabe K, Watanabe A, Midorikawa Y, Yamamoto S, Ihara S, et al. Identification of soluble NH_2-terminal fragment of Glypican-3 as a serological marker for early-stage hepatocellular carcinoma, Cancer Res 2004;64:2418-2423.
24. Yoon SK, Lim NK, Ha SA, Park YG, Choi JY, Chung KW, et al. The human cervical cancer oncogene protein is a biomarker for human hepatocellular carcinoma. Cancer Res 2004;64:5434-5441.
25. Yokoyama Y, Kuramitsu Y, Takashima M, Iizuka N, Toda T, Terai S, et al. Proteomic profiling of proteins decreased in hepatocellular carcinoma from patients infected with hepatitis C virus. Proteomics 2004;4:2111-2116.
26. Kim W, Oe Lim S, Kim JS, Ryu YH, Byeon JY, Kim HJ, et al. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin Cancer Res 2003;9:5493-5500.
27. Steel LF, Shumpert D, Trotter M, Seeholzer SH, Evans AA, London WT, et al. A strategy for the comparative analysis of serum proteomes for the discovery of biomarkers for hepatocellular carcinoma. Proteomics 2003;3: 601-609.
28. Steel LF, Mattu TS, Mehta A, Hebestreit H, Dwek R, Evans AA, et al. A proteomic approach for the discovery of early detection markers. Dis Markers 2001;17:179-189.
29. Liang CR, Leow CK, Neo JC, Tan GS, Lo SL, Lim JW, et al. Proteome analysis of human hepatocellular carcinoma tissues by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 2005;5: 2258-2271.
30. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2003;2:1096-1103.
31. Chignard N, Beretta L. Proteomics for hepatocellular carcinoma marker discovery. Gastroenterology 2004;127:s120-s125.
32. Villanueva J, Martorella AJ, Lawlor K, Philip J, Fleisher M, Robbins RJ, et al. Serum peptidome patterns that distinguish metastatic thyroid carcinoma from cancer-free controls are unbiased by gender and age. Mol Cell Proteomics 2006;5:1840-1852.
33. Scarlett CJ, Smith RC, Saxby A, Nielsen A, Samra JS, Wilson SR, et al. Proteomic classification of pancreatic adenocarcinoma tissue using protein chip technology. Gastroenterology 2006;130:1670-1678.
34. Won Y, Song HJ, Kang TW, Kim JJ, Han BD, Lee SW. Pattern analysis of serum proteome distinguishes renal cell carcinoma from other urologic diseases and healthy persons. Proteomics 2003;3:2310-2316.
35. Zhu XD, Zhang WH, Li CL, Xu Y, Liang WJ, Tien P. New serum biomarkers for detection of HBV-induced liver cirrhosis using SELDI protein chip technology. World J Gastroenterol 2004;10:2327-2329.
36. Poon TC, Yip TT, Chan AT, Yip C, Yip V, Mok TS, et al. Comprehensive proteomic profiling identifies serum proteomic signatures for detection of hepatocellular carcinoma and its subtypes. Clin Chem 2003;49:752-760.
37. Soresi M, Magliarisi C, Campagna P, Leto G, Bonfissuto G, Riili A, et al. Usefulness of alpha-feroprotein in the diagnosis of hepatocellular carcinoma. Anticancer Res 2003;23:1747-1753.
38. Sherman M. Alphafetoprotein: an obituary. J Hepatol 2001;34:603-605.
39. Song BC, Chung YH, Kim JA, Choi WB, Suh DD, Pyo SI, et al. Transforming growth factor-beta1 as a useful serologic marker of small hepatocellular carcinoma. Cancer 2002;94:175-180.
40. Miura N, Maeda Y, Kanbe T, Yazama H, Takeda Y, Sato R, et al. Serum human telomerase reverse transcriptase messenger RNA as a novel tumor marker for hepatocellular carcinoma. Clin Cancer Res 2005;11:3205-3209.
41. Honda M, Kaneko S, Kawai H, Shirota Y, Kobayashi K. Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 2001;120:955-966.
42. Lee IN, Chen CH, Sheu JC, Lee HS, Huang GT, Chen DS, et al. Identification of complement C3a as a candidate biomarker in human chronic hepatitis C and HCV-related hepatocellular carcinoma using a proteomics approach. Proteomics 2006;6:2865-2873.

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Transgenic expression of osteoactivin in the liver attenuates hepatic fibrosis in rats

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Abstract

The role of osteoactivin (OA) in liver fibrogenesis remains unclear. After feeding wild-type (WT) and OA transgenic (OA-Tg) rats a choline-deficient, L-amino acid-defined (CDAA) diet for 12 weeks, we evaluated liver fibrosis. Hepatic fibrosis and expression of α-smooth muscle actin protein in OA-Tg rats were reduced in comparison to WT rats. Our examination of the expression of 31,100 genes by microarray analysis identified 177 and 256 genes that were upregulated and downregulated, respectively, by at least twofold in OA-Tg rat livers in comparison to WT rat livers. Of these genes, we confirmed a significant downregulation in the expression levels of tissue inhibitor of metalloproteinase-1 and -2, type I collagen, and platelet-derived growth factor receptor-α and -β in the livers of OA-Tg rats. These results indicate that transgenic OA expression attenuates the development of hepatic fibrosis in association with the suppression of specific genes involved in its pathogenesis.

Keywords: Osteoactivin; Choline-deficient L-amino acid-defined diet; Hepatic fibrosis; TIMP; PDGF receptor

Hepatic fibrosis is a common response seen in chronic liver diseases, which ultimately leads to cirrhosis, a major public health problem worldwide. Hepatic fibrosis can be attenuated by treatment of the cause of liver injury such as anti-viral therapy and abstinence from alcohol [1]. There is no efficient treatment, however, for most causes of chronic liver disease and no effective direct treatment for hepatic fibrosis in a clinical setting.

Hepatic stellate cells (HSC) are currently thought to be primarily responsible for hepatic fibrosis. In response to hepatic injury, quiescent HSCs are activated to become myofibroblastic cells, which produce cytokines and matrix proteins like transforming growth factor (TGF)-β and tissue inhibitor of matrix metalloproteinase (TIMP)-1 [2]. In addition, the signal transduction pathways activated in HSC by hepatic injury have suggested targets for the direct treatment of hepatic fibrosis in animal models [3,4].

A wide spectrum of pathological features are observed in non-alcoholic fatty liver disease (NAFLD), ranging from fatty liver to steatohepatitis and hepatic fibrosis, and hepatocellular carcinoma (HCC). In the choline-deficient, L-amino acid-defined (CDAA) diet rat model, liver steatosis occurs within one week. Hepatic fibrosis appears one month after administration of CDAA diet, with cirrhosis appearing after three or four months and HCC develops twelve to fifteen months after administration of CDAA [5]. To represent these pathological features, rats fed a CDAA

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diet is recognized one of the animal models for chronic liver disease, especially NAFLD.

Osteoactivin (OA) cDNA was originally isolated from osteopetrotic bone [6]. OA, also known as glycoprotein nonmetastatic melanoma protein B or dendritic cell-associated heparan sulfate proteoglycan-integrin ligand, is a type I transmembrane glycoprotein that influences the adhesion and migration of select cell types, including fibroblasts [7]. In addition, we previously reported the OA gene as a molecule that is differentially expressed in the livers of rats administered a CDAA diet [8]. The molecular mechanism by which OA functions in liver disease, however, has yet to be fully clarified. This study sought to determine the role of OA in hepatic fibrosis using transgenic rats that express OA in the liver after CDAA diet-induction of hepatic fibrosis.

Materials and methods

Generation of transgenic rat. A rat OA cDNA fragment, encoding the entire open reading frame from nucleotide 110 to 1917, was amplified by polymerase chain reaction (PCR) and cloned into the *Eco*RI site of the pLG-1 expression vector, which contains the human serum amyloid P (SAP) promoter and a rabbit β-globin non-coding exon/intron [9]. After digestion of the resulting plasmid with *Hin*dIII and *Xho*I, the 3.8-kb SAP-OA gene fragment was microinjected into fertilized Sprage Dawley (SD) rat eggs to produce transgenic rats (OA-Tg rats). Animal protocols were approved by the ethical committee of the Faculty of Medicine, University of Miyazaki.

RNA isolation, Northern blotting and RT-PCR. The total RNA was extracted from liver tissue, separated on agarose gels and transferred onto nylon membranes. To detect the OA mRNA transcript, we used radiolabeled 1808-bp rat OA cDNA and 483-bp rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as probes. We also validated the OA gene expression levels by semi-quantitative reverse transcription (RT)-PCR. The total RNA was reverse transcribed using Molony murine leukemia virus reverse transcriptase (TaKaRa, Tokyo, Japan) in the presence of random hexamers. The following primers were then used: 5′-ACACTG CAGCCTGACAACTCA-3′ and 5′-TTTGCCCTTGACCACGTTC-3′ for rat OA and 5′-ACTCTACCCACGGCAAGTTCA-3′ and 5′-GG CAGTGATGGCATGGACT-3′ for rat GAPDH. The reverse-transcribed mixture was amplified by PCR in a 25 μl volume. PCRs were initially denatured, then cycled at 94 °C for 30 s, 52 °C (OA) or 59 °C (GAPDH) for 30 s, and 72 °C for 30 s. Thirty cycles served to amplify OA or GAPDH. PCR products of OA and GAPDH were examined by agarose gel electrophoresis and visualized with ethidium bromide. Densitometric analysis examined the amount of PCR products semi-quantitatively by measuring absorbance on a Bio-1D apparatus (M&S Instruments Trading Inc., Tokyo, Japan).

Hepatic fibrosis induced by a 12-week CDAA diet. Ten-week-old male SD rats bearing or lacking the SAP-OA gene (OA-Tg and WT, respectively) were used. SD rats were obtained from Japan SLC (Yokohama, Japan). After at least a one-week acclimation period on a standard diet, OA-Tg and WT (control) rats were switched to the CDAA diet (Dyets, PA) as a model of hepatic fibrosis. Rats were analyzed after a 12-week administration of the CDAA diet.

Histological and immunohistochemical analysis, and quantification of hepatic hydroxyproline content. Tissue samples were fixed in 10% phosphate-buffered formaldehyde, then embedded in paraffin and stained with either Azan or Sirius Red. Three liver fragments (>1 cm^2 each) were randomly taken from the right, median, and left lobes of each rat liver for morphometric studies. Sirius Red (Sirius Red 80; MUTO PURE CHMICALS Co., Tokyo, Japan) staining was performed as described previously [10]. To analyze fibrosis present in Sirius Red-stained sections, the red-stained areas were measured on a video-screen display in a blinded manner using a digital image analyzer pixs2000Pro (Inotech, Hiroshima, Japan) [4]. Three fields were selected randomly from each of three sections per sample; samples from six rats from each group were examined. Thus, a total of 54 fields were analyzed for each group. After signals were quantified, we calculated the mean area of fibrosis. Immunohistochemical analysis of α-smooth muscle actin (α-SMA) (Dako Japan, Kyoto, Japan) was performed and hepatic hydroxyproline content was determined as previously described [11,12].

DNA microarray analysis. RNA samples were reverse-transcribed and copied into dsDNA. *In vitro* RNA transcription was then performed to incorporate biotin-labeled ribonucleotides into the cRNA transcripts. The resulting cRNA samples were hybridized to a Rat Genome 230 2.0 Array (Affmetrix Inc., CA). Detailed protocols for the analysis of microarray data have been previously described [13,14]. We excluded genes that were not expressed or those that were expressed at levels below the cutoff level for detection in both OA-Tg and WT rat livers. These data were transferred to GeneSpring software (Silicon Genetics, CA) for additional analysis.

Western blotting. Liver tissues were homogenized in Tissue Protein Extraction Reagent (Pierce Biotechnology, IL). Ten micrograms of sample was subjected to SDS–polyacrylamide gel electrophoresis and transferred onto Immobilon P membranes (Millipore Corp., MA). The following primary antibodies were used for analysis: monoclonal anti-α-SMA antibody (SIGMA), polyclonal anti-TIMP-1 antibody, polyclonal anti-platelet-derived growth factor receptor (PDGFR)-α antibody (Santa Cruz Biotechnology, CA) and monoclonal anti-β-actin antibody (Dako Japan). Bound antibody was detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham Biosciences, Buckinghamshire, UK). Proteins were then visualized using the ECL Western blotting detection kit (Amersham).

Statistical analysis. Results are presented as means ± standard deviation. Statistical analysis was performed using Statview J-4.5 software (Abacus Concepts, Inc., CA). Differences were assessed by the Kruskal–Wallis analysis and/or the Mann–Whitney *U* test. The significance level was set at $P < 0.05$.

Results

Osteoactivin expression in the various organs

Northern blot analysis revealed that rat OA mRNA was strongly expressed in the lung and spleen and weakly expressed in the brain, heart, and liver. Expression was

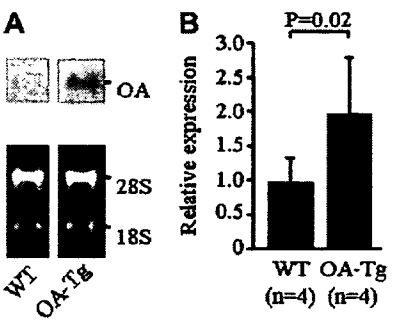

Fig. 1. Osteoactivin (OA) expression in the liver. (A) Northern blot analysis of OA expression in rat at 10 weeks of age. (B) Quantitative determination of OA mRNA using semiquantitative RT-PCR is shown as an average of four experiments ± standard deviation. The mean relative intensity in WT rat livers with normal diet administration was normalized to a value of 1.

Table 1
Serum biochemical markers in osteoactivin transgenic rats

	Normal diet normal rat ($n = 3$)	CDAA12W	
		WT ($n = 6$)	OA-Tg ($n = 6$)
Glucose (mg/dl)	157.3 (7.77)	217.2 (32.4)[a]	257.5 (68.8)[a]
ALT (IU/L)	65.7 (10.7)	53.0 (14.1)[a]	120.8 (30.1)[a,b]
LDH (IU/L)	1951.7 (501.3)	1173.2 (516.8)	1974.0 (811.3)
ALP (IU/L)	799.7 (44.0)	739.5 (331.6)	647.2 (129.5)
Triglyceride (mg/dl)	187.3 (64.0)	40.0 (8.44)[a]	105.5 (64.7)[c]
Total cholesterol (mg/dl)	70.7 (7.51)	62.5 (12.9)[a]	100.2 (13.1)[a,b]

Results (and standard deviation of the mean) from 6 rats/group at the end of feeding period were shown. WT, wild-type; OA-Tg, osteoactivin transgenic.
[a] $P < 0.05$ versus the normal rat with normal diet.
[b] $P < 0.01$ versus WT rats with CDAA diet.
[c] $P < 0.05$ versus WT rats with CDAA diet.

absent from the kidneys of non-transgenic littermates and SD rats as previously reported [8]. The expression levels of OA in the lung, spleen, brain, and heart of OA-Tg rats were similar to those seen in non-transgenic littermates (data not shown). In contrast, the OA expression in the liver of OA-Tg rats were higher than those seen in non-transgenic littermates, and the levels in those was twofold higher by semi-quantitative RT-PCR analysis (Fig. 1).

Attenuation of CDAA diet-induced hepatic fibrosis in OA-Tg rats

Serum levels of ALT, triglyceride, and total cholesterol in OA-Tg rats were significantly higher than those seen in WT rats (Table 1). In contrast, the serum levels of glucose, LDH and ALP did not significantly differ between OA-Tg and WT rats.

Hepatic fibrosis was induced in both OA-Tg and WT rats by a 12-week CDAA diet administration. Histological analysis with Azan and Sirius Red staining demonstrated CDAA-induced severe fibrosis in the livers of WT rats (Fig. 2A and C). In contrast, fibrosis was not as prominent in the livers of CDAA-treated OA-Tg rats (Fig. 2B and D). This reduction in the severity of the fibrosis was observed in all areas of the liver, with no significant differences noted between the different lobes. In addition, the number of CDAA-induced fibrosis areas and the hydroxyproline content of OA-Tg rat livers were significantly lower in comparison to those seen in WT rats (Fig. 2E and F).

Overexpression of OA decreased the number of activated hepatic stellate cells

Although the number of activated HSCs expressing α-SMA (a marker of activated HSC) increased in WT rats fed the CDAA diet, overexpression of OA dramatically reduced the number of α-SMA-positive cells observed in

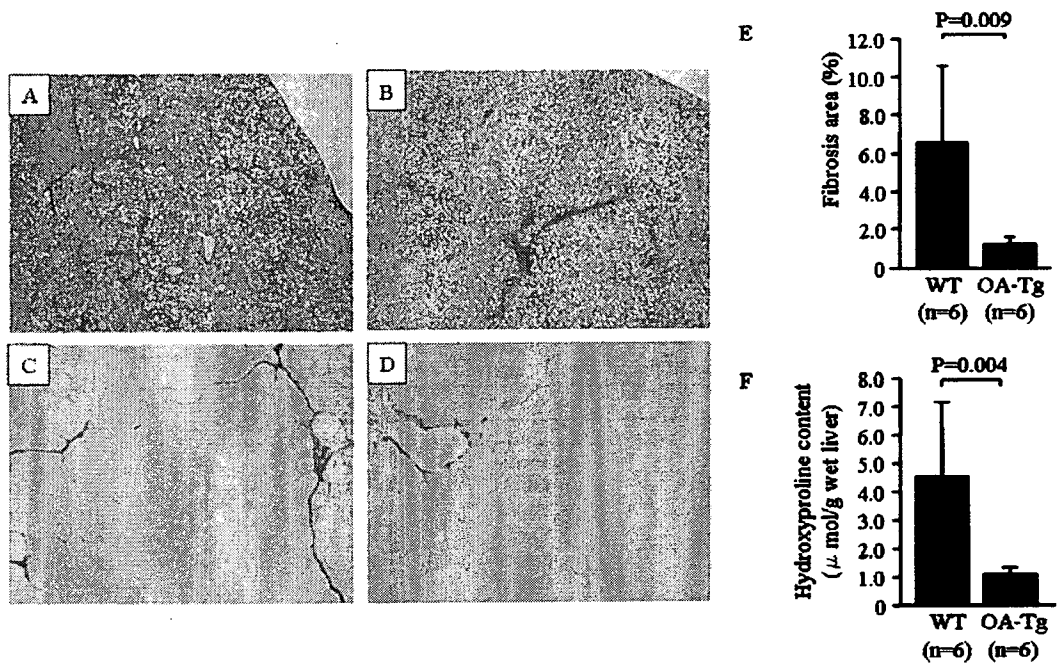

Fig. 2. Histological features of the liver in non-transgenic (A,C) or transgenic (B,D) rats after 12 weeks CDAA diet administration. Representative Azan (A,B) or sirius red (C,D) staining of the liver tissue (original magnification 40× (A,B), 100× (C,D)). In addition, quantitative evaluation of hepatic fibrosis in osteoactivin transgenic (OA-Tg) ($n = 6$) and non-transgenic (wild-type; WT, $n = 6$) rats are shown. (E,F) Morphometric quantification of the percentage of fibrosis areas and the hydroxyproline content in the livers of OA-Tg rats were significantly lower than those seen in WT rats, respectively.

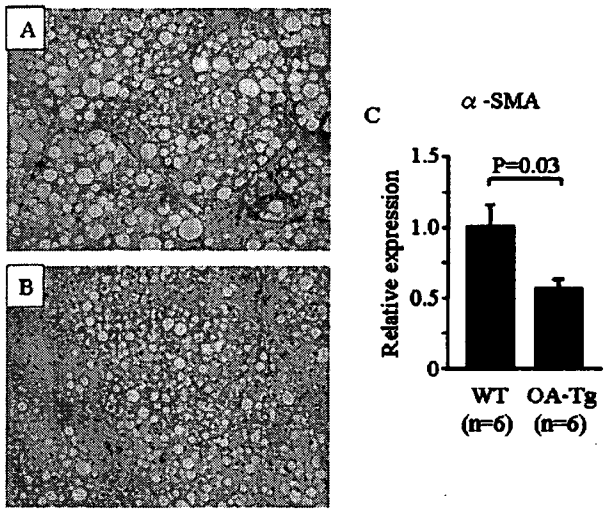

Fig. 3. Quantitative evaluation of hepatic α-smooth muscle actin (α-SMA) expression after 12 weeks CDAA diet administration. (A,B) Representative immunohistochemistry examining α-SMA expression in the livers of wild-type (A) or osteoactivin transgenic (B) rats (original magnification 100×). (C) Quantitative expression of hepatic α-SMA was determined by western blot analysis using an image analyzer. Results are shown as the averages with standard deviations. Expression of hepatic α-SMA in osteoactivin transgenic (OA-Tg) rats was significantly lower than that seen in wild-type (WT) rats.

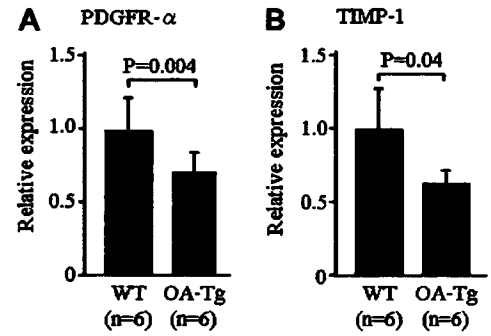

Fig. 4. Quantitative evaluation of hepatic platelet-derived growth factor receptor-α (PDGFR-α) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression after 12 weeks CDAA diet administration. (A,B) Quantitative expression of hepatic PDGFR-α and TIMP-1 were determined by Western blot analysis using an image analyzer. Results are shown as the averages with standard deviations. Expression of hepatic PDGFR-α (A) and TIMP-1 (B) in osteoactivin transgenic (OA-Tg) rats were lower than those seen in wild-type (WT) rats.

the liver (Fig. 3A and B). By western blot analysis, we also observed a significant decrease in α-SMA protein expression in OA-Tg rats in comparison to WT rats (Fig. 3C).

Gene expression profiles in the liver using DNA microarray analysis

The total RNA, isolated from the livers, of six OA-Tg or six WT rats, were mixed equally and hybridized in parallel to two identical oligonucleotide arrays. cRNA poles of OA-Tg or WT rats were used in each array. The statistical analysis identified 15692 transcripts which were expressed above the cutoff level for detection in both OA-Tg and WT rats. Of those genes, 177 transcripts displayed significantly increased signal intensities in mixed RNA derived from OA-Tg rat liver in compared to that from WT rat liver; 256 transcripts exhibited reduced signal intensities (data not shown). Of these genes, only 59 and 121 genes, respectively, have functional annotations in public databases. We selected from those 180 transcripts genes associated with fibrosis using the NetAffx Analysis Center database. Table 2 displays the numerical and descriptive analysis of these differentially expressed fibrosis genes; genes that presented with a fold change higher than 2.0 (upregulated) or lower than 0.5 (downregulated). Type I collagen, PDGF receptor (PDGFR)-α and -β, and TIMP-1 and -2, which are factors known to contribute to the development of hepatic fibrosis, were markedly downregulated in the livers of OA-Tg rats in comparison to WT rats. We also confirmed the downregulation of

Table 2
List of the fibrosis associating genes in osteoactivin transgenic rat after the administration of 12 weeks CDAA diet[a]

Gene name	Symbol	Fold change over controls	Accession No.
Collagen, type 1, alpha 1	Col1a1	0.225	BI285575
Suppressor of cytokine signaling 2	Socs2	0.359	NM_058208
Latent transforming growth factor beta binding protein 1	Ltbp1	0.38	NM_021587
Platelet derived growth factor receptor, alpha polypeptide	Pdgfra	0.391	AI232379
Tissue inhibitor of metalloproteinase 2	Timp2	0.397	BM388843
Serine proteinase inhibitor, clade H, member 1	Serpinh1	0.399	BI285495
Collagen, type III, alpha 1	Col3a1	0.406	BI275716
Tissue inhibitor of metalloproteinase 1	Timp1	0.406	NM_053819
Hypothetical gene supported by NM_031525	Pdgfrb	0.436	BM389426
Prostaglandin E receptor 1	Ptger1	0.467	AA945828
Fibrillin 1	Fbn1	0.478	BM389019
Serine protease inhibitor, Kazal type 1	Spink1	2.522	NM_012674

[a] Selected genes associated with fibrosis those are altered in osteoactivin transgenic (OA-Tg) rat. Fold changes in the livers of OA-Tg rats are compared to those in the livers of wild-type rats after a 12-week administration of the CDAA diet. Prior to DNA microarray analysis, mRNAs were mixed from each group ($n = 6$, each) as described in Materials and methods.

PDGFR-α mRNA expression in the liver by RT-PCR (data not shown). In addition, the expression of PDGFR-α and TIMP-1 in the liver observed by Western blot analysis was clearly decreased in the OA-Tg rats in comparison to WT rats (Fig. 4).

Discussion

Expression of the rat OA gene restricted to osteoblasts in bone [6]. Haralanova-Ilieva et al. demonstrated that OA is expressed at high levels in normal and inflammatory liver macrophages, suggesting a role for this protein in acute liver injury [15]. OA is also expressed in dendritic cells and tumor cells. OA is thought to induce fibroblasts activation [16]. OA overexpression increases the invasiveness and metastatic potential of rat hepatoma cells both *in vitro* and *in vivo* [8]. Although these results indicate that OA has multiple effects on different cell types, the function of OA in hepatic fibrosis remains unclear. In this study, we provide the first direct evidence that transgenic expression of OA in the liver inhibited hepatic fibrosis in rats fed a CDAA diet for 12 weeks.

Transgenic expression of OA was driven by the SAP promoter, which induces specific gene expression in hepatocytes, but not non-parenchymal cells [17]. Although transgenic expression of OA was only induced by the SAP promoter at low levels, hepatocyte expression of OA in OA-Tg rats was able to reduce hepatic fibrosis in association with a decrease in the number of activated HSCs. HSC activation typically induces a myoblastic, fibroblastic phenotype of these cells. Overexpression of OA in fibroblasts, but not mouse myoblasts, induced the expression of matrix metalloproteinase (MMP)-3. Thus, OA may function as an activator for fibroblasts that have infiltrated denervated skeletal muscle [16]. In this study, however, transgenic expression of OA had no effect on MMP-3 expression in the liver (data not shown). OA may have different roles in liver HSCs and muscle fibroblasts. OA may induce different effects in different disease processes, such as hepatic fibrogenesis and denervation.

No apparent changes in matrix-related gene expression have been demonstrated in OA transgenic mice [16]. OA-Tg rats did not display any apparent abnormalities. After taking the CDAA diet for 12 weeks, however, serum ALT and total cholesterol (TC) levels were higher in OA-Tg rats than those seen in WT rats. Although ALT increases with increasing severity of hepatitis, it is not indicative of hepatic fibrosis severity; high levels of ALT are instead observed in the early phases of liver disease in rats fed a CDAA diet [8]. Decreasing levels of TC have been linked to increasing severity of liver disease [18], suggesting that low ALT and TC levels in WT rats fed a CDAA diet indirectly indicate the severity of hepatic fibrosis. Analysis of OA activity in the different stages of fibrosis is required to identify the molecular foundation of this effect on fibrotic pathogenesis.

OA, which localizes to the cell surface and lysosomal membranes [7], can also be secreted from cells [6]. The role of OA expression in chronic liver disease remains unclear; in humans, OA expression is not detectable in normal liver tissue [8]. OA transcripts become detectable in cirrhotic non-tumorous liver tissue that surrounds HCC foci. OA mRNA expression is strongly induced in the livers of rats fed the CDAA diet for 4–12 weeks. Additional exogenous expression of OA, however, attenuates hepatic fibrosis. Although we did not identify the cells expressing OA and could not evaluate the amount of OA secreted, the secreted form of OA released by hepatocytes may negatively regulate activated HSCs. Further examination, including the effect of secreted OA on the activation of non-parenchymal cells, is required.

Both PDGFR-α and PDGFR-β were downregulated by exogenous OA expression (Table 2). Expression of the PDGFR correlates well with the extent of hepatic fibrosis [19]. While PDGFR-α is constitutively expressed in quiescent HSC, PDGFR-β expression is induced as cells undergo myofibroblastic changes [20]. Although the data concerning PDGFR-α and PDGF-β expression patterns are conflicting [21], targeting PDGFR-α signaling is an attractive potential therapeutic intervention in hepatic fibrosis. We also demonstrated the downregulation of suppressor of cytokine signaling (SOCS)-2, latent transforming growth factor-β binding protein (LTBP)-1, prostaglandin E receptor 1, and fibrillin 1 in OA-Tg rat livers in comparison to WT rat livers. TGF-β activity requires the proteolytic cleavage of LTBP, a microfibril-associated protein that interacts with fibrillin [22]. A number of (myo)fibroblastic cell subpopulations in the liver synthesize fibrillin-1, whose expression is induced by TGF-β1. These results indicate that the attenuation of hepatic fibrosis by transgenic OA expression is closely linked to the suppression of these genes.

The attenuation of PDGF signaling in the livers of OA-Tg rats would theoretically be accompanied by decreases in the expression of multiple target genes of PDGF signaling in comparison to WT rats. In this study, expression of extracellular signal-regulated kinase (ERK)-7, one of the targets of PDGF signaling, in OA-Tg rat liver was below detectable levels, excluding ERK-7 from the analysis. ERK-7 levels, however, were decreased twofold in the livers of OA-Tg rats in comparison to WT rats. These results suggest that transgenic expression of OA in the liver functionally attenuates PDGF signaling. Despite the many differences at the mRNA level identified by cDNA array analysis, our study only examined a small fraction of the liver transcriptome; additional important genes may not have been identified in our analysis. Further analysis of mRNA samples derived from specific cell populations, such as hepatocytes, HSCs, Kupffer cells, and endothelial cells, from both OA-Tg and WT rats may be required.

In conclusion, transgenic rats expressing OA exclusively in hepatocytes exhibited attenuated hepatic fibrosis in response to a CDAA diet. The potential of OA to increase

the risk of HCC tumor invasiveness and metastasis may limit the use of this molecular target in the treatment of liver cirrhosis. Further investigation using a secreted form of OA will be necessary and may lead to the development of novel therapeutic approaches to hepatic fibrosis.

Acknowledgments

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References

[1] S. Guerret, A. Desmoulière, P. Chossegros, A.M. Costa, C. Badid, C. Trépo, J.A. Grimaud, M. Chevallier, Long-term administration of interferon-alpha in non-responder patients with chronic hepatitis C: follow-up of liver fibrosis over 5 years, J. Viral. Hepat. 6 (1999) 125–133.

[2] E. Ueberham, R. Löw, U. Ueberham, K. Schönig, H. Bujard, R. Gebhardt, Conditional tetracycline-regulated expression of TGF-beta1 in liver of transgenic mice leads to reversible intermediary fibrosis, Hepatology 37 (2003) 1067–1078.

[3] J. George, D. Roulot, V.E. Koteliansky, D.M. Bissell, In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis, Proc. Natl. Acad. Sci. USA 96 (1999) 12719–12724.

[4] E. Borkham-Kamphorst, J. Herrmann, D. Stoll, J. Treptau, A.M. Gressner, R. Weiskirchen, Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis, Lab. Invest. 84 (2004) 766–777.

[5] D. Nakae, H. Yoshiji, Y. Mizumoto, K. Horiguchi, K. Shiraiwa, K. Tamura, A. Denda, Y. Konishi, High incidence of hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats, Cancer Res. 52 (1992) 5042–5045.

[6] F.F. Safadi, J. Xu, S.L. Smock, M.C. Rico, T.A. Owen, S.N. Popoff, Cloning and characterization of osteoactivin, a novel cDNA expressed in osteoblasts, J. Cell Biochem. 84 (2001) 12–26.

[7] S. Shikano, M. Bonkobara, P.K. Zukas, K. Ariizumi, Molecular cloning of a dendritic cell-associated transmembrane protein, DC-HIL, that promotes RGD-dependent adhesion of endothelial cells through recognition of heparan sulfate proteoglycans, J. Biol. Chem. 276 (2001) 8125–8134.

[8] M. Onaga, A. Ido, S. Hasuike, H. Uto, A. Moriuchi, K. Nagata, T. Hori, K. Hayash, H. Tsubouchi, Osteoactivin expressed during cirrhosis development in rats fed a choline-deficient, L-amino acid-defined diet, accelerates motility of hepatoma cells, J. Hepatol. 39 (2003) 779–785.

[9] T. Toyonaga, O. Hino, S. Sugai, S. Wakasugi, K. Abe, M. Shichiri, K. Yamamura, Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver, Proc. Natl. Acad. Sci. USA 91 (1994) 614–618.

[10] A. López-De León, M. Rojkind, A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections, J. Histochem. Cytochem. 33 (1985) 737–743.

[11] H. Yoshiji, S. Kuriyama, J. Yoshii, Y. Ikenaka, R. Noguchi, T. Nakatani, H. Tsujinoue, H. Fukui, Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats, Hepatology 34 (2001) 745–750.

[12] J.F. Woessner, The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid, Arch. Biochem. Biophys. 93 (1961) 440–447.

[13] D.J. Lockhart, H. Dong, M.C. Byrne, M.T. Follettie, M.V. Gallo, M.S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton, E.L. Brown, Expression monitoring by hybridization to high-density oligonucleotide arrays, Nat. Biotechnol. 14 (1996) 1675–1680.

[14] T.R. Golub, D.K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J.P. Mesirov, H. Coller, M.L. Loh, J.R. Downing, M.A. Caligiuri, C.D. Bloomfield, E.S. Lander, Molecular classification of cancer: class discovery and class prediction by gene expression monitoring, Science 286 (1999) 531–537.

[15] B. Haralanova-Ilieva, G. Ramadori, T. Armbrust, Expression of osteoactivin in rat and human liver and isolated rat liver cells, J. Hepatol. 42 (2005) 565–572.

[16] T. Ogawa, T. Nikawa, H. Furochi, M. Kosyoji, K. Hirasaka, N. Suzue, K. Sairyo, S. Nakano, T. Yamaoka, M. Itakura, K. Kishi, N. Yasui, Osteoactivin upregulates expression of MMP-3 and MMP-9 in fibroblasts infiltrated into denervated skeletal muscle in mice, Am. J. Physiol. Cell Physiol. 289 (2005) C697–C707.

[17] S. Mochida, T. Yoshimoto, S. Mimura, M. Inao, A. Matsui, A. Ohno, H. Koh, E. Saitoh, S. Nagoshi, K. Fujiwara, Transgenic mice expressing osteopontin in hepatocytes as a model of autoimmune hepatitis, Biochem. Biophys. Res. Commun. 317 (2004) 114–120.

[18] C. Cicognani, M. Malavolti, A.M. Morselli-Labate, L. Zamboni, C. Sama, L. Barbara, Serum lipid and lipoprotein patterns in patients with liver cirrhosis and chronic active hepatitis, Arch. Intern. Med. 157 (1997) 792–796.

[19] M. Pinzani, S. Milani, H. Herbst, R. DeFranco, C. Grappone, A. Gentilini, A. Caligiuri, G. Pellegrini, DV. Ngo, R.G. Romanelli, P. Gentilini, Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis, Am. J. Pathol. 148 (1996) 785–800.

[20] L. Wong, G. Yamasaki, R.J. Johnson, S.L. Friedman, Induction of beta-platelet-derived growth factor receptor in rat hepatic lipocytes during cellular activation in vivo and in culture, J. Clin. Invest. 94 (1994) 1563–1569.

[21] K. Breitkopf, C. Roeyen, I. Sawitza, L. Wickert, J. Floege, A.M. Gressner, Expression patterns of PDGF-A, -B, -C and -D and the PDGF-receptors alpha and beta in activated rat hepatic stellate cells (HSC), Cytokine 31 (2005) 349–357.

[22] Z. Isogai, R.N. Ono, S. Ushiro, D.R. Keene, Y. Chen, R. Mazzieri, N.L. Charbonneau, D.P. Reinhardt, D.B. Rifkin, L.Y. Sakai, Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein, J. Biol. Chem. 278 (2003) 2750–2757.

慢性肝炎に対する肝庇護療法

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ポイント

- 肝炎ウイルスによる慢性肝炎において，第一の治療目標は，生体からの肝炎ウイルスの排除である．
- 肝炎ウイルスが排除不可能である場合，肝炎の鎮静化，残存肝機能保護の目的で肝庇護薬が使用される．
- 肝庇護薬により肝炎の鎮静化が可能ならば，肝硬変への移行や肝癌合併にも有効と考えられるが，効果には限界がある．
- 難治性のウイルス肝炎では，肝庇護薬にてできるだけ肝機能を保ちつつ，新規抗ウイルス薬の開発を待つ．

ウイルス肝炎に対する根本的な治療は，抗ウイルス薬投与により生体内から肝炎ウイルスを完全に排除することであるが，その根本的治療が無効であった場合や，副作用により治療の継続が不可能な場合には，いわゆる肝庇護薬による治療が行われる．この肝庇護薬には肝細胞膜保護作用，肝再生作用，組織修復作用，胆汁分泌作用，抗脂肪作用など多岐にわたる薬理作用をもつものが含まれ，肝炎患者への投与により肝内の抗炎症効果を発揮しトランスアミナーゼの低下が期待できる．

本稿では，肝庇護薬として代表的なグリチルリチン製剤(強力ネオミノファーゲンC®：SNMC)，ウルソデオキシコール酸(UDCA)，漢方薬について，薬理効果や作用機序などについて述べる．

グリチルリチン製剤 (強力ネオミノファーゲンC®：SNMC)

強力ネオミノファーゲンC®(SNMC)は，グリチルリチン，システイン，グリシンを配合した注射薬で，グリチルリチンがその主な有効成分と考えられている．その薬理作用としては，①抗炎症作用，②免疫調節作用，③肝細胞障害抑制作用，④ウイルス増殖抑制作用などが挙げられる．グリチルリチンの抗炎症効果は，ホスホリパーゼA_2やリポキシゲナーゼに直接結合し，これらアラキドン酸代謝系酵素のリン酸化を阻害することによる[1]．また，グリチルリチンはその代謝産物であるグリチルレチン酸となり，肝組織中のα_2(I)コラーゲン遺伝子の転写を直接抑制し，抗線維化作用を示すといわれている．グリチルリチンがHCVコア蛋白の誘導する活性酸素を抑制する作用も報告さ

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れている.

生体免疫系に関する作用としては，T細胞活性化調節作用，インターフェロン-γ誘起作用のほか，NK細胞活性化作用，胸腺外Tリンパ球分化増強作用などが報告されている．また，SNMCは抗アレルギー作用も有するため，蕁麻疹の治療にも用いられる.

実際の臨床研究では，グリチルリチンは血清ALT値を低下させ，肝線維化の進展や肝細胞癌の発癌率を低下させることが知られている[2]．慢性肝疾患に対して一般に初期投与では，40～60 m*l*を連日静脈内注射あるいは点滴静注し，血清トランスアミナーゼ値を観察していく．SNMCは週3日投与より，週6日投与のほうが効果的である．効果があれば，週2～3回の投与に減らし維持していく．SNMCは用量依存的にALTを低下させるといわれており，効果がみられない場合には，100 m*l*まで増量し肝炎の沈静化を図り，安定したら徐々に減量していく．SNMC 100 m*l*，8週間投与で，肝細胞壊死像，細胞浸潤像などの肝組織学的所見が改善したという報告がある.

副作用として，血清カリウム値の低下が認められることがあり，注意を要する.

ウルソデオキシコール酸(UDCA)

ウルソデオキシコール酸(UDCA：ウルソ®)の肝障害改善における作用機序としては，①肝細胞保護作用，②利胆作用，③免疫調節作用などが考えられている．UDCA経口投与により，内因性胆汁酸の回腸末端からの吸収が抑制され，患者の胆汁中の胆汁酸組成がUDCA優位となる．肝細胞毒性の強いリトコール酸やケノデオキシコール酸などの脂溶性胆汁酸を，親水性胆汁酸であるUDCAに置き換えることで，肝障害の軽減が期待できる．また，UDCAには強い利胆作用があることが報告されているが，うっ滞した疎水性胆汁酸が肝内に蓄積し引き起こす肝障害に対しUDCAを投与することは，その利胆作用により肝内から疎水性胆汁酸の排泄を促進させる効果があると考えられる．そして，UDCAの肝細胞保護作用としてUDCA自体が直接細胞膜を安定化させることも想定されている．種々の抗体や薬剤で培養肝細胞のアポトーシスの誘導を試みた研究では，UDCAはミトコンドリア膜を安定化させて細胞のアポトーシスを抑制した.

さらに，UDCAは生体内で免疫抑制・抗炎症作用を発揮する可能性が示唆されている．UDCAは免疫グロブリンの産生や，Tリンパ球のIL-2産生を抑制することが知られている．原発性胆汁性肝硬変(PBC)患者肝細胞においてはMHC class I分子のみならず，免疫担当細胞以外では例外的にMHC class IIも発現しているが，UDCAはMHC class IとclassIIの両者の発現を減少させると報告されている.

ほかに，UDCAには腸管からのエンドトキシン吸収抑制作用やミトコンドリア起因性酸化ストレスを制御して肝細胞のアポトーシスを抑制することなども想定されている.

UDCAの慢性胆汁うっ滞性の肝疾患に対する治療は，1987年Poupon らがPBCでの有用性を示してから[3]，PBCに対しては現在第一選択薬として臨床の場で使用されている．慢性肝疾患に対し一般に600～900 mg/日の投与が行われている．UDCA投与で肝組織の炎症像の改善は認められるものの，肝線維化の改善はわずかといわれている．1985～1992年までの臨床研究をまとめた解析でも，肝組織所見の改善は認めないものの，血清ALT，AST，ALP，ビリルビン値は改善すると報告されている.

漢方薬

約20年前から小柴胡湯をはじめとする漢方薬も，慢性肝疾患に対し用いられるようになった．

小柴胡湯は7種類の構成生薬よりなり，バイカリン，サイコサポニン，グリチルリチンなどが有効成分といわれている．小柴胡湯には薬理作用として，①肝血流量低下抑制作用，②肝再生促進作用，③肝線維化抑制作用(伊東細胞の活性化や細胞外マトリックスの産生を抑制)，④免疫調整作用，⑤免疫複合体除去作用，⑥抗アレルギー作用，⑦抗炎症作用，⑧活性酸素抑制作用があるとされている．臨床面においてはALT低下作用や病理組織像改善など，慢性肝炎に対する有効性を示す報告がなされている[4]．肝炎が軽度である例や高齢者に長期にわたり使用する際に小柴胡湯が推奨されている．また，漢方薬の使用において，肝硬変への移行が懸念される場合には柴胡桂枝湯を，体力のある実証タイプには大柴胡湯を，反対に虚証タイプには補中益気湯を，というように，症例にあわせて漢方薬の処方を考慮すべきであろう．

小柴胡湯に関する報告では，インターフェロン治療無効例に対し，小柴胡湯投与により開始4，8，12，24週後で，血清ALT値が開始時と比較し有意に低下し，44.5％の症例で有用との評価がある．

肝硬変で顕著にみられる急激に起こる筋肉の痙攣を伴う疼痛(こむらがえり)に対しては，芍薬甘草湯が有効である．

小柴胡湯の使用に際しては，重大な副作用としてまず，間質性肺炎の合併を観察する必要がある．特に，インターフェロン製剤との併用は間質性肺炎の誘因となるため，禁忌とされている．また，血小板数が15万/mm³以下の患者に対しては，肝硬変に移行していることも疑われるため禁忌とされている．さらに，偽アルドステロン症やミオパシーの合併にも注意が必要である．

おわりに

慢性肝炎で肝障害が進行しており，インターフェロンなどの抗ウイルス療法が無効な例や使用できない例に対しては，上記の肝庇護薬での治療に頼らざるを得ないのが現状である．肝庇護薬でトランスアミナーゼ値を低下させておくことは，肝炎の沈静化により肝硬変への移行を防止するとともに，肝癌の合併を抑止する働きがあると考えられる．

SNMCやUDCAは，インターフェロンやラミブジンといった抗ウイルス薬と比べ安価で副作用も少なく手軽に使用できるが，その肝炎鎮静効果や発癌抑制効果には限界があり，患者にはやはり第一にウイルス排除を目指すべきことを強調すべきであろう．難治性のウイルス肝炎患者に対しては，これら肝庇護薬を的確に投与し肝炎の進行を抑えつつ，また新たな抗ウイルス薬の出現を待つのが，現在できうる最善策の1つと考えられる．

文　献

1) Shimoyama Y, et al : Physiological correlation between glycyrrhizin, glycyrrhizin-binding lipoxygenase and casein kinase II. FEBS Lett 391 : 238-242, 1996
2) Kumada H, et al : Long-term treatment of chronic hepatitis C with glycyrrhizin [stronger neo-minophagen C (SNMC)] for preventing liver cirrhosis and hepatocellular carcinoma. Oncology 62(Suppl 1) : 94-100, 2002
3) Poupon R, et al : Is ursodeoxycholic acid an effective treatment for primary bilialy cirrhosis? Lancet 1 : 834-836, 1987
4) Hirayama C, et al : A multicenter randomized controlled clinical trial of Shosaiko-to in chronic active hepatitis. Gastroenterol Jpn 24 : 715-719, 1989

特集II　ウイルス肝炎の免疫学的機序と治療

C型肝炎の獲得免疫*

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Key Words：cytotoxic T cell, helper T cell, regulatory T cell, dendritic cell, immuno-suppression

はじめに

C型肝炎は，C型肝炎ウイルス(hepatitis C virus：HCV)そのものが直接肝細胞を破壊して発症するのではなく，患者自身の免疫細胞がHCVに感染した肝細胞を排除する目的で肝細胞を傷害する，という免疫応答により発症すると考えられている．HCVを特異的に認識する免疫細胞はC型肝炎患者の肝臓内や末梢血中に存在するが，これらの細胞はHCVを排除し感染を終息させようとする生体防御にかかわる一方で，肝炎の慢性化や重症化にも関与している．

急性HCV感染はB型肝炎ウイルス(hepatitis B virus：HBV)感染と異なり，約70%が後に持続感染に至る．高頻度にHCVが持続感染する理由として，免疫応答が十分に誘導される前にHCVが急速に増殖することや，HCV遺伝子が多様に変異すること，HCV蛋白が生体の免疫を抑制することなどが報告されている．また，HCV感染初期における患者の免疫応答の強さがC型肝炎の転帰を大きく左右するといわれており，HCVに対する免疫応答を観察し詳細に検討することは，HCVの排除や肝炎の終息を目的とした新規治療法の確立，さらにはHCV感染の予防法の開発に大きな意味をもつ．

本稿では，HCVに対する獲得免疫応答を中心に，C型肝炎における肝障害の発症機序や，HCVが生体免疫から逃避して持続感染に至る機序につき，これまでに報告されている論文をもとに解説する．

C型肝炎における免疫応答

HCVが生体に感染すると，まず生体内では非特異的免疫応答が生じる．HCV感染初期は，HCVに感染した肝細胞や，感染を認知した形質細胞様樹状細胞(plasmacytoid dendritic cell)などから産生されるインターフェロン(IFN)-α/β(I型IFN)によりウイルスの増殖抑制が試みられる．I型IFNは細胞内で2'-5'オリゴアデニル酸合成酵素などを誘導しHCVの増殖を抑制するほか，樹状細胞などの抗原提示細胞においてヒト白血球抗原(human leukocyte antigen：HLA)class I分子の細胞表面への出現を増強させる作用や，ナチュラルキラー(natural killer：NK)細胞，細胞障害性T細胞(CTL)などの免疫細胞を活性化させる作用などの免疫応答増強作用を有する．IFN-αにより活性化したNK細胞はHCVに感染した肝細胞を認識し障害を起こす．肝細胞が障害を受けることにより刺激を受けた骨髄系樹状細胞(myeloid dendritic cell)は，NK細胞や，NK細胞とT細胞の両者の性質をもち肝臓に多く存在するNKT細胞を活性

* Acquired immune responses in HCV infection.

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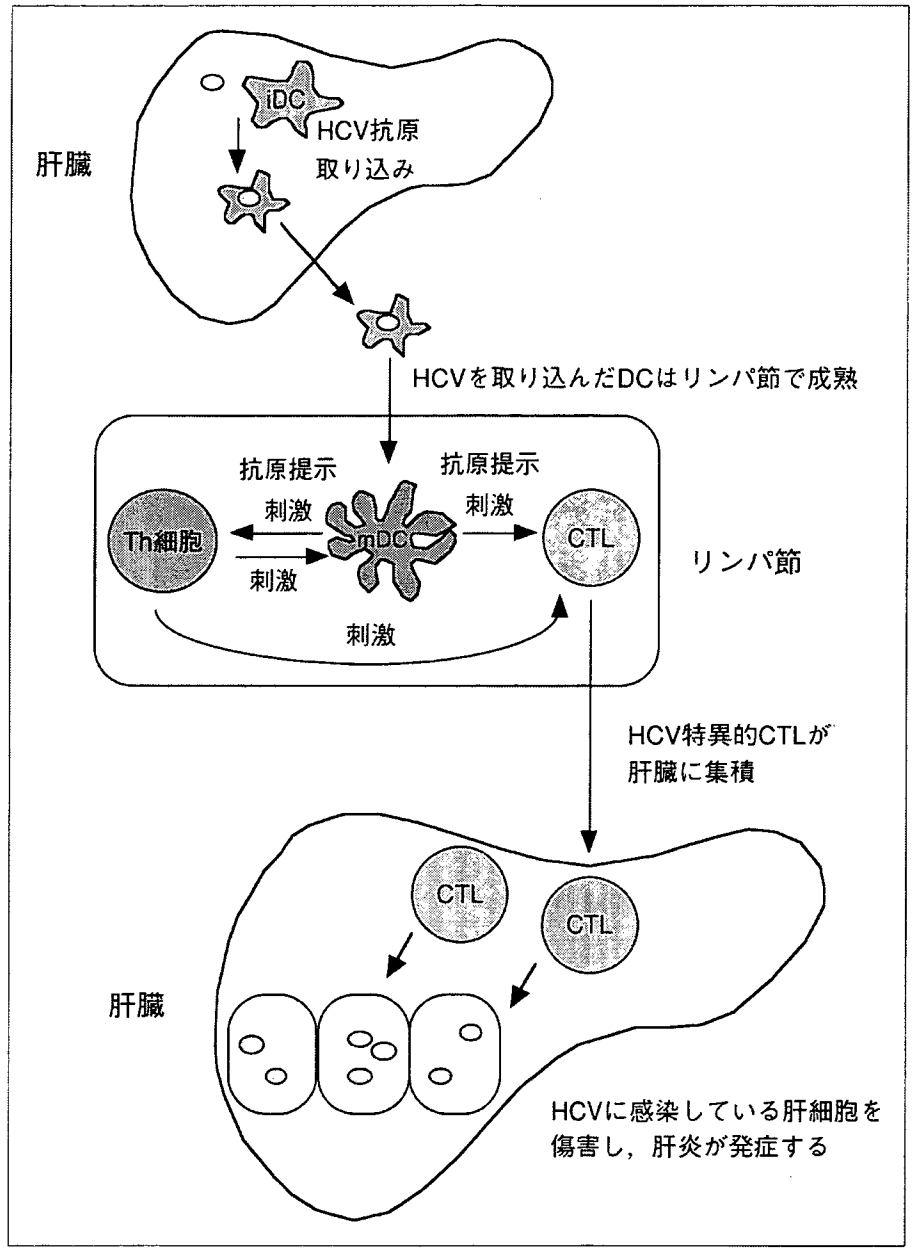

図1　HCV特異的免疫応答の誘導と肝炎の発症機序
iDC：未成熟樹状細胞，mDC：成熟樹状細胞，Th細胞：ヘルパーT細胞，
CTL：細胞障害性T細胞

化し，それらの細胞はIFN-γを多量に分泌する．さらに，IFN-γはマクロファージの活性化を増強し，局所の炎症反応を増強する．

HCV感染において，上記の非特異的免疫応答によりHCVが十分に生体から排除できない場合，獲得免疫が誘導され，さらなるウイルスの排除が試みられる．獲得免疫の中では，ヘルパーT(Th)細胞やCTLがHCV排除に重要な役割を担っている．

図1に，HCV特異的細胞免疫が誘導される過程を示す．まず，骨髄系樹状細胞はHCV抗原を取り込むと所属リンパ節に遊走し，そこで表面に共刺激分子などの発現を増強させて成熟した樹状細胞となり，T細胞を活性化する．樹状細胞は，表面のHLA class II分子上に提示されたHCV抗原を認識する未感作Th細胞を刺激し活性化させる．それにより活性化したTh細胞は，CD40リガンドを表出し，さらに腫瘍壊死因子(tumor necrosis factor：TNF)-αなどのサイトカインを分泌することで樹状細胞をさらに成熟，活性化させる．主に骨髄系樹状細胞が産生するインターロイキン(interleukin：IL)-12は，感作されたTh細

胞をⅠ型Th(Th1)細胞に分化，誘導し，その後Th1細胞はIL-2やIFN-γを産生してCTLやNK細胞を刺激し，活性化や増殖を促す．それらにより未感作CTLは樹状細胞が提示するHCV抗原を認識し，初めて感作される．感作し活性化されたHCV特異的CTLは，リンパ節を離れ肝臓に到達し，HCVに感染した肝細胞の表面にあるHLA class Ⅰ分子上に提示されたHCV抗原を認識して，感染細胞の細胞死を誘導することによりHCVの排除を試みる．しかし，同時にこの肝細胞死は肝炎発症の原因にもなる．

初感染時におけるHCV排除にはHCV特異的CTLの存在が重要であると報告されており，HCV持続感染例でもCTL応答がHCVの増殖を抑制していることが示唆されている．われわれはHLA B44に拘束性のCTLエピトープを報告して[1)2)]以来，合成ペプチドやELISPOT法などを用いて，HCVのCTLエピトープをこれまでに10数種類発見し報告してきた[3)4)]．これらのエピトープのアミノ酸配列に相当する合成ペプチドで末梢リンパ球を刺激しCTL活性を測定したところ，HCV急性感染例や感染後3年以内の比較的早期の慢性肝炎患者ではHCV特異的CTL応答が検出されやすかったのに対し，10年以上経過した例においてはCTL応答がほとんどみられなかった．急性感染後のHCV自然排除例では，血中HCV RNA量の減少に伴いCTL応答も低下していった．一方で，HCV排除例では，排除後数十年という長期間にわたりHCVに対する$CD4^+$や$CD8^+$T細胞の応答が認められることも報告されている[5)]．HCV感染時に適度な細胞性免疫応答が生じた場合には，HCVは完全に排除される．しかし，HCV感染では生体での免疫応答が一般に十分ではないため，HCVによる急性肝炎では肝障害の程度が比較的軽く，感染が持続化しやすいと考えられる．それに対して，非常に強い免疫応答が誘導されると，劇症肝炎などの重症な肝障害をひき起こす可能性がある．このように，HCV量と免疫応答の強さとのバランスによりさまざまな程度の肝障害が起こると考えられる．

また，C型慢性肝炎で活性化したHCV特異的CTLは肝臓に集積しており，ウイルスの増殖抑制と肝障害に関与していることや，HCV量の少ないC型慢性肝炎患者では末梢血中にもHCV特異的CTLが検出されることが報告されている．CTLが検出されないのは，多量のHCVがT細胞を消費している可能性も考えられている[6)]．HCV特異的CTL応答のみられた進行したC型慢性肝炎患者では，有意に血清ALT値が高値を示していたが，他の臨床像や病理組織像とは関連が少なかったことも報告された[7)]．

近年，HCVとインフルエンザウイルスの免疫交叉性が指摘された[8)]．強い肝炎を伴った患者では，肝炎が軽度である場合には認められないHCV NS3領域の1,073～1,081番目のアミノ酸に対する強いCTL応答がみられたが，この部分はインフルエンザウイルスのノイラミニダーゼ(neuraminidase)の配列と交叉反応性を有していた．この部分のノイラミニダーゼペプチドに対するCTL応答は，重度の肝障害を有するC型肝炎患者だけに認められた[8)]．このことは，ある種のインフルエンザに罹患した患者にHCVが感染した場合，強い肝炎が起こる可能性が示唆され，このようなCTLの免疫交叉反応性が，C型肝炎の発症や肝炎の炎症の強さに影響することも考えられる．また，この部分のアミノ酸変異は，HCVにとってCTLの認識から逃れることができる一方，HCVの生存や複製に必要なプロテアーゼ活性が失われる可能性があり，この免疫原性の高いエピトープ領域にアミノ酸変異のあるHCV株は出現しにくいと考えられ[9)]，この部分を標的とした治療法の開発も期待できる．

HCV特異的CTLの肝細胞障害機序

HCV特異的CTLは表面のT細胞受容体により，HCV感染肝細胞の表面に存在するHLA class Ⅰ分子とその上に提示されている8～11個のアミノ酸よりなるHCV抗原ペプチドを認識し，細胞表面に孔を形成するパーフォリンや，細胞をアポトーシスに陥らせるグランザイムを標的であるHCV感染細胞に放出して細胞死をひき起こす．

また，活性化したCTLはFasリガンドやTNF-αの発現も増強しており，標的細胞を障害する．パーフォリンはほとんどすべての細胞に細胞障害活性を示すのに対し，FasリガンドやTNF-αはそれらの受容体をもち感受性がある細胞のみに効力を発揮する．正常の肝細胞はFasリガンドや

TNF-αに抵抗性を示すが，肝炎患者の肝組織中の炎症が強い部位ではFas抗原やTNF受容体の発現が増強しており，FasリガンドやTNF-αに対しての感受性も高まっていることが考えられる．Fas-Fasリガンド系やTNF-αの系を介した細胞障害活性はパーフォリンと比較すると細胞障害効率は低いものの，活性化したCTLはこれらの系を介して局所の炎症などで感受性が高まったHCV非感染細胞をも障害すると考えられる[10]．

IFN治療による免疫応答の変化

IFN-αは，抗ウイルス作用のほか，major histocompatibility complex(MHC)class I分子の発現増強，NK細胞活性増強，CTLのアポトーシス制御，樹状細胞の活性化といった免疫増強作用など，多彩な生物学的作用を有することが報告されている．

C型肝炎の治療としてIFN-α製剤は広く使用されているが，C型急性肝炎に対しては発症後4か月以内でのIFN単独療法でHCVの排除率は98%と報告されている[11]のに対し，C型慢性肝炎ではペグ化IFN-α製剤とリバビリンの併用でもsustained viral response(SVR)率は56%にとどまっており[12]，急性と慢性では治療効果に差異が認められる．われわれは，IFN治療を行ったC型肝炎患者において，IFNが実際に患者末梢単核球のHCV全域を網羅するように作製した297個のHCV合成ペプチドに対するIFN-γ産生を，IFN投与前後で経時的にELISPOT法で検討したところ，C型急性肝炎ではIFN投与後HCV量が減少していくにつれ，各ペプチドに対する応答も低下する傾向がみられた[4]．また，C型慢性肝炎患者に対してIFN/リバビリン併用療法前後で免疫応答を測定したところ，CTL活性は治療開始後，いったん増強する傾向がみられたものの，その後の変化はさまざまであり，SVRと免疫応答の強さとは関連が認められなかった．

ほかにも，C型急性肝炎における免疫応答の検討で，HCVを自然排除した症例ではHCV特異的ヘルパーT細胞応答は増強するのに対し，IFN治療でSVRが得られた症例では増強しないことも報告されている[13]．

このように，IFN治療は，末梢のHCV特異的免疫応答を増強することでHCVの排除を促すということに否定的な報告がある一方，IFNによるCTL活性の増強とSVRには関連がみられるという報告もあり[14][15]，現在のところ一定の見解が得られておらず，今後もさらなる検討が必要である．

C型肝炎における液性免疫応答

HCV pseudo-particleの開発により患者血清中のHCV抗体の中和活性を*in vitro*で測定することが可能となった．チンパンジーや急性肝炎での検討では，HCV排除と中和抗体の検出は一致しないことや，進行したC型慢性肝炎では中和抗体価と臨床経過や病理組織像は関連がみられないこと[16]などが報告され，現在のところ，液性免疫とC型肝炎の病態との関連は少ないと考えられている．

HCVの免疫逃避と免疫抑制機構

1．HCVのアミノ酸変異による生体免疫機構からの逃避

HCVは，アミノ酸変異を起こさせやすいRNAポリメラーゼの作用や高い増殖複製能により，生体内にさまざまなタイプのHCVが存在するというクアシスピーシスを形成し，宿主の免疫監視機構からの逃避を試みている[17][18]．また，クアシスピーシスはさまざまな細胞への感染を可能にすることや薬剤に対する耐性の獲得にも影響を及ぼすと考えられる．

(1)液性免疫からの逃避

HCV E2領域内の超可変領域(hypervariable region 1：HVR1)におけるアミノ酸変異はきわめて多様であり，生体で産生されるHCVに対する中和抗体による認識から逃れている可能性がある一方で，チンパンジーでのHCV感染実験からは，HVR1に変異を認めなくても持続感染に発展していることが報告されており，E2領域の変異が感染の持続化に重要であるかは一定の見解が得られていない．

(2)細胞性免疫からの逃避

T細胞のエピトープ領域の変異は，HCV感染肝細胞の排除を妨げることにより感染の持続化に寄与すると考えられる．これまでの研究では，感染HCVのクローニングにより，$CD8^+$ CTLの認

識から逃れるアミノ酸変異が認められている．また，HLA-DRB1拘束性でNS3由来のペプチドを認識するTh1タイプのT細胞に対し，このエピトープ内の1つのアミノ酸に変異が起こると，分泌するサイトカインがTh1タイプからTh2タイプに変化したことが報告されたが[19]，一般にこのようにTh2タイプの免疫応答が生体内で優位になると持続感染が成立しやすくなる．

HCVのCTLエピトープにおけるアミノ酸変異がみられた症例では，変異した配列はもとの配列と比較しCTLに認識されにくく，さらにCTLを効果的に誘導できないと報告されている[20][21]．CTLエピトープの変異は，より強いエフェクター機能を有するCTLをターゲットにして起こり，持続感染に寄与することも想定される．

一方，HCV感染に対する初期のCTL応答は多様であり，一つのエピトープの変異のみで持続化は説明できないとの指摘もある．エスケープミュータントは持続感染の原因というより，持続感染の結果をみている可能性も否定できない．

2. HCV感染によるT細胞の機能抑制

HCVに対する免疫応答として，急性期には多様なHCV特異的T細胞応答が認められるが，慢性化するとその応答は劇的に減弱してしまう．肝内には多数のHCV特異的$CD8^+$T細胞が存在してはいるものの，HCVを排除できない．また，C型慢性肝炎患者にはウイルスや細菌感染などの合併も多くみられ，以前から生体での免疫力の低下が想定されてきた．近年，HCV蛋白が能動的に生体の免疫機構を抑制しているという報告が多数なされるようになり注目を浴びている．

HCV特異的CTLはエフェクター機能の低下が指摘されている．C型慢性肝炎患者の末梢リンパ球には，機能の発現に重要なCD3ζ鎖の発現が低下していると報告されている．HBV特異的CTLに比しHCV特異的CTLは明らかにパーフォリンの発現量が少ないとされ，これも機能低下を示す一因とされている．

C型急性肝炎時の$CCR7^-CD8^+$T細胞(メモリー・エフェクター細胞)は細胞障害活性が低下しているが，これにIL-2を加えるとエフェクター機能を有する細胞に変化することから，T細胞が活性化する際のIL-2の欠乏がCTLの機能低下の主な原因であるとの報告もある[22]．循環しているHCVコア蛋白がIL-2産生のシグナル伝達の抑制に関与していることも想定されている．C型慢性肝炎患者では，HCV特異的$CD4^+$T細胞の存在は認められるが抗原特異的な増殖能は抑制されている．

また，HCV感染肝細胞より遊離し末梢血中に存在するHCVコア蛋白は，T細胞のgC1qRと結合することで，T細胞の増殖や活性，IFN-γ産生能を阻害することが報告された．HCVコア蛋白は血中にナノグラムの単位で存在しており，gC1qRと結合するには十分量と考えられるが，肝組織内ではさらに高濃度のコア蛋白が存在していると想定され，肝浸潤リンパ球に少なからず影響を与えていると推測される．

HCV NS4A/B蛋白は，細胞内で小胞体からゴルジ体への輸送を妨げることにより，HLA class I分子の細胞上への発現を抑制することが報告された[23]．これにより，HCV特異的$CD8^+$T細胞がHCV感染肝細胞を認識しにくくなり，HCVの感染持続化に繋がることも考えられる．

さらに，肝臓には類洞内皮細胞やKupffer細胞といった免疫に関与する細胞が存在するが，それらは成熟した樹状細胞とは異なり，ウイルス抗原は提示するもののCD80やCD86といった共刺激分子に乏しいためT細胞を十分に刺激できないばかりか，かえって免疫寛容を誘導してしまうことも考えられている[24]．

これら種々の原因により，Th細胞やCTLの十分な増殖や活性化が起こらないことが，HCVの持続感染を導く原因と考えられる．

3. HCV特異的制御性T細胞の関与

生体の免疫応答を抑制する要因の一つとして，抗原を特異的に認識してIL-10やTGF-βを産生する制御性T(regulatory T：Treg)細胞が注目されている．C型慢性肝炎患者においては，Treg細胞と考えられる$CD4^+CD25^+$T細胞のfrequencyが高く，この細胞集団は直接T細胞の機能を抑制し，これがHCV特異的細胞性免疫の質的，量的な抑制をひき起こして，肝炎の持続化に寄与していると想定されている[25]．C型肝炎患者ではHCV抗原刺激に特異的なIL-10やTGF-β産生が有意にみられるが，HCVコア蛋白に特異的なTreg細胞がC型慢性肝炎の末梢血から分離誘導され，

この細胞が産生するIL-10がHCV感染の持続化に関与すると報告された[26]．また，C型肝炎患者の肝内にはIL-10を産生するHCV特異的CCR7$^-$CD8$^+$Treg細胞が存在し，肝内に多数集積しているHCV特異的CCR7$^-$CD8$^+$メモリーT細胞の機能を抑制することも報告された[27]．さらに，HCV NS3やNS4蛋白は，C型肝炎患者のみならず，健常者の末梢単核球からもIL-10の産生を促しIL-12の分泌を抑制させ，さらに樹状細胞の分化成熟化を抑制するとの報告もあり，細胞性免疫の活性化抑制の一つの機序として興味深い[28][29]．

以上のようにTreg細胞のHCV感染持続化への関与が強く示唆されているが，まだ不明な点も多くさらなる検討が必要である．

おわりに

C型肝炎に対して患者の臨床経過とともに患者の免疫応答を詳細に測定するモニター法が普及し，C型肝炎に対する生体防御機構とC型肝炎ウイルス(HCV)による免疫回避機構が少しずつ解明されてきた．しかしながら，全容解明にはさらなる検討が必要である．

HCV排除に向けた治療成績の向上には，HCV特異的免疫応答を高めていく方法を検討すべきであろう．

文　献

1) Kita H, Moriyama T, Kaneko T, et al. HLA B44-restricted cytotoxic T lymphocytes recognizing an epitope on hepatitis C virus nucleocapsid protein. Hepatology 1993 ; 18 : 1039-44.
2) Kita H, Hiroishi K, Moriyama T, et al. A minimal and optimal cytotoxic T cell epitope within hepatitis C virus nucleoprotein. J Gen Virol 1995 ; 76 (Pt 12) : 3189-93.
3) Kaneko T, Nakamura I, Kita H, et al. Three new cytotoxic T cell epitopes identified within the hepatitis C virus nucleoprotein. J Gen Virol 1996 ; 77 (Pt 6) : 1305-9.
4) Hakamada T, Funatsuki K, Morita H, et al. Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epitopes by ELISpot assay using peptides with human leukocyte antigen-A*2402-binding motifs. J Gen Virol 2004 ; 85(Pt 6) : 1521-31.
5) Wertheimer AM, Miner C, Lewinsohn DM, et al. Novel CD4$^+$ and CD8$^+$ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. Hepatology 2003 ; 37 : 577-89.
6) Hiroishi K, Kita H, Kojima M, et al. Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. Hepatology 1997 ; 25 : 705-12.
7) Rothman AL, Morishima C, Bonkovsky HL, et al. Associations among clinical, immunological, and viral quasispecies measurements in advanced chronic hepatitis C. Hepatology 2005 ; 41 : 617-25.
8) Urbani S, Amadei B, Fisicaro P, et al. Heterologous T cell immunity in severe hepatitis C virus infection. J Exp Med 2005 ; 201 : 675-80.
9) Söderholm J, Ahlén G, Kaul A, et al. Relation between viral fitness and immune escape within the hepatitis C virus protease. Gut 2006 ; 55 : 266-74.
10) Ando K, Hiroishi K, Kaneko T, et al. Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. J Immunol 1997 ; 158 : 5283-91.
11) Jaeckel E, Cornberg M, Wedemeyer H, et al. Treatment of acute hepatitis C with interferon alfa-2b. N Engl J Med 2001 ; 345 : 1452-7.
12) Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002 ; 347 : 975-82.
13) Rahman F, Heller T, Sobao Y, et al. Effects of antiviral therapy on the cellular immune response in acute hepatitis C. Hepatology 2004 ; 40 : 87-97.
14) Tsai SL, Sheen IS, Chien RN, et al. Activation of Th1 immunity is a common immune mechanism for the successful treatment of hepatitis B and C : tetramer assay and therapeutic implications. J Biomed Sci 2003 ; 10 : 120-35.
15) Freeman AJ, Marinos G, Ffrench RA, et al. Intrahepatic and peripheral blood virus-specific cytotoxic T lymphocyte activity is associated with a response to combination IFN-alpha and ribavirin treatment